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Age-specific sensitivity of sperm length and testes size to developmental temperature in the bruchid beetle

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Abstract

In an era of global warming, the negative effects of temperature stress on fertility could intensify predicted losses of biodiversity. Male fertility is particularly sensitive to temperature stress, yet we have an incomplete understanding of when, during reproductive ontogeny, spermatogenesis is most affected. Here, we used a temperature-switch protocol to identify when during development temperature affects the expression of sperm length and testes size in the bruchid beetle *Callosobruchus maculatus*. Egg-to-adult development took place at 17°C, 27°C or 33°C for either the full duration of pre-imago development or it was switched at different stages during development. Full development at 17°C or 33°C resulted in significantly shorter sperm than at 27°C. However, when developing larvae were switched to higher or lower temperatures, we observed switch-specific phenotypic expression of sperm length and testes size. Our key finding was that sperm length was sensitive to high-temperature stress during the early stages of ontogeny and to low-temperature stress during the latter stages of ontogeny. Such age-specific developmental sensitivity suggests that infertility resulting from transient heat stress (i.e. heatwaves) could be mitigated by age-related developmental heterogeneity within populations. Those individuals able to avoid severe effects of heat stress on fertility, through serendipitously being at less-sensitive stages of development, could potentially compensate for the loss of fertility experienced by those individuals at more sensitive stages of development.

Introduction

Insect ontogeny encompasses the processes by which an egg transforms into a sexually mature adult. Despite being described as a series of stages, the process is continuous (Heming, 2003; Gullan & Cranston, 2014). Like many biological processes, insect ontogeny is strongly influenced by temperature whereby developmental processes are greatest (in terms of rates or successful completion) around a given thermal optimum and decline as critical thermal maxima and minima are reached (Ratte, 1985; Angilletta Jr., 2009; Gullan & Cranston, 2014). The relationship between insect development and temperature is relatively well-studied (Ratte, 1985). However, recently there has been renewed interest in the consequences of sub-lethal developmental temperature on fertility. Elevated temperatures experienced during development have been shown to reduce both male and female fertility, placing populations at greater risk of extinction in the face of global climate change (David *et al*., 2005; Colinet *et al.,* 2015; Sage *et al*., 2015; Sales *et al*., 2018; Walsh *et al*., 2019; Parratt *et al*., 2020; Zwoinska *et al*., 2020).

The relationship between developmental temperature and reproductive ontogeny has its roots in environmental sex determination (Warner & Shine, 2008). In mosquitoes (*Aedes stimulans*), males reared at high temperatures lack the usual complement of testes, vasa differentia and seminal vesicles, but instead express ovaries, oviducts and spermathecae (Horsfall & Anderson, 1961). It appears that the primordia within the male gonadal rudiments follow female developmental trajectories when temperatures are sufficiently high (Horsfall & Anderson, 1964). Less extreme, but no less important, effects of developmental temperature on spermatogenesis were reported by Cohet (1973) and David (1971) studying *Drosophila*. They were amongst the first to report reduced fertility resulting from impairments to cyst elongation during spermatogenesis in response to high temperatures (Rohmer *et al*., 2004; David *et al*., 2005). Subsequently, a growing number of studies have reported sperm length to be affected by developmental temperature across a variety of taxa, including *Scathophaga stercoraria* flies (Blanckenhorn & Hellriegel, 2002), *Arianta arbustorum* snails (Minoretti *et al*., 2013), *Poecilia reticulata* fishes (Breckels & Neff, 2014), *Callosobruchus maculatus*

beetles (Vasudeva *et al*., 2014) and *Plodia interpunctella* moths (Iossa *et al*., 2019).

Despite a growing list of studies reporting the developmental temperature to affect spermatogenesis, few studies have attempted to identify when during larval development spermatogenesis is sensitive to temperature stress. Thermal tolerance, and the plasticity therein, are not static over the physiological age of the insect (Bowler & Terblanche, 2008). For example, in the blowfly, *Calliphora erythrocephala*, egg and early-stage larvae are less tolerant to heat stress than later developmental stages (Davison, 1969). Thus, in the current context, given spermatogenesis is a protracted process, in which the production of functional sperm during the later stages of development relies on the successful organogenesis of the testes during earlier stages of development, then it is likely the process will be more or less sensitive to temperature stress during different stages of ontogeny (Boivin *et al*., 2005; Chevrier *et al*., 2019). However, very little is known about the sensitivity of spermatogenesis to thermal stress when applied at the different stages of development.

Here we use a temperature-switch protocol to evaluate when during development testes and sperm are sensitive to changes in temperature. Traditionally, this approach has been used to identify thermally sensitive phases of development in species with temperature-dependent sex determination (Pieau & Dorizzi, 1981; Shine, 2006; Valenzuela, 2008). We utilize the observation that in the bruchid beetle, *Callosobruchus maculatus*, the sperm of males that developed at 17°C or 33°C are smaller than those of males grown at 27°C (Vasudeva *et al*., 2014). Thus, by rearing larvae at these two temperature extremes for various periods of time before switching them to 27°C, we aim to identify when during development, sperm length and testes size are affected by developmental temperature.

Materials and methods

Study populations

The *Callosobruchus maculatus* beetles used in this study came from a large, outbred population (~5000 adults) cultured for 24 generations on moth beans (*Vigna aconitifoli*) in an insectary maintained at 27°C, ~35% relative humidity, and a 16h light:8h dark photoperiod. The parental stock population originated from Niamey, Niger, and had been kept on black-eyed beans (*Vigna ungliculata*) for >250 generations at the University of Lincoln, UK. In these trials, moth beans were used to limit variation in larval density as typically one adult only emerges from each seed irrespective of the number of eggs oviposited on them (Vasudeva, 2014).

Approximately 1000 adults (estimated by mass) were housed with 200 g of moth beans (~7500 beans) for one hour at 27°C and ~35% RH, to oviposit. The egg-laden seeds were then separated into 6 triple-vent Petri dishes (110 mm, Fisherbrand, www.fisher.co.uk), at a density of approximately 1500 moth beans per Petri dish. These were placed into incubators (Lucky Reptile Herp Nursery II, The Reptile Shop, UK), with two incubators (replicates) set at either 17°C, 27°C or 33°C. Assayed adults were all 24–48 h post-eclosion. The beetles used in this study were maintained under conditions normal to their life cycle and the University of Lincoln's principles and standards for good ethical practice in research were followed.

Temperature-switch experiments

Egg-laden beans were switched from either 27°C to 17°C (and vice versa; 17°C to 27°C) or from 27°C to 33°C (and vice versa; 33°C to 27°C) at various stages of larval development. Experimental controls in our assays were those that completed 100% of their development at either 17°C, 27°C or 33°C, where no temperature switching occurred. Previously, we reported a large effect of temperature on larval development (Vasudeva *et al*., 2018), with egg-to-adult development taking on average 88 days to complete at 17°C, whilst those incubated at 33°C took ~ 20 days (Fig. 1). Thus, temperature-switch periods were established as time spent in development as a percentage relative to total larval development time elapsed at the starting temperature. We determined the developmental stages (i.e. larval instars) for carrying out the larval switching from previous work (see Vasudeva *et al*., 2018).

Prior to adult emergence, the beans containing the developing larvae were placed into individual cells of a 25 cell replidish (www.fishersci.co.uk, Sterilin™) and sealed with a clear-glass lid. Repli-dishes were checked for adult emergence every 24 h. Upon eclosion, approximately 20 males were euthanized by freezing at −5°C in an Eppendorf tube and stored for morphological measurements. Elytra length (mm) was measured as a proxy for body size.

The bi-lobed testes of *C. maculatus* are located on either side of the gut. Testes size was quantified from freshly defrosted males under an Olympus SZH10 dissection microscope. For each specimen, the entire reproductive tract was dissected free from other tissues and organs in a drop of insect saline. From here the testes were dissected free and then placed on a microscope slide under a 30 μL droplet of insect saline Digital images of the testes were captured using a Moticam 2000 camera linked to a Nikon Eclipse E600 microscope at (10X magnification). The 2D area of the testes was measured using '*ImageJ*' (Schneider *et al.,* 2012) image analysis software's segmented tool by drawing landmarks around the spherical lobes (Vasudeva, 2014). Elytra length was measured using a line tool on ImageJ. An elytron (hardened fore wing) was removed from the adult using watchmaker's forceps then imaged under a dissection microscope. A straight line was drawn along the centre of the elytra, using the line tool, to calculate the length (mm).

Following image capture of testes, the testes were ruptured using the fine tip of a watchmaker's forceps (Fisherbrand) within a small drop (30 μL) of pre-prepared insect saline. A sub-sample of this droplet was transferred to a microscope slide and examined for sperm using a Nikon Eclipse E600 microscope, under dark-field illumination and a 10X objective. By randomly scanning the different parts of the prepared slide, images of intact sperm were captured using a Moticam 2000 camera. Total sperm length (mm) was quantified from the digitized images using the segmented hand tool in '*ImageJ*' image analysis software. Ten randomly selected males

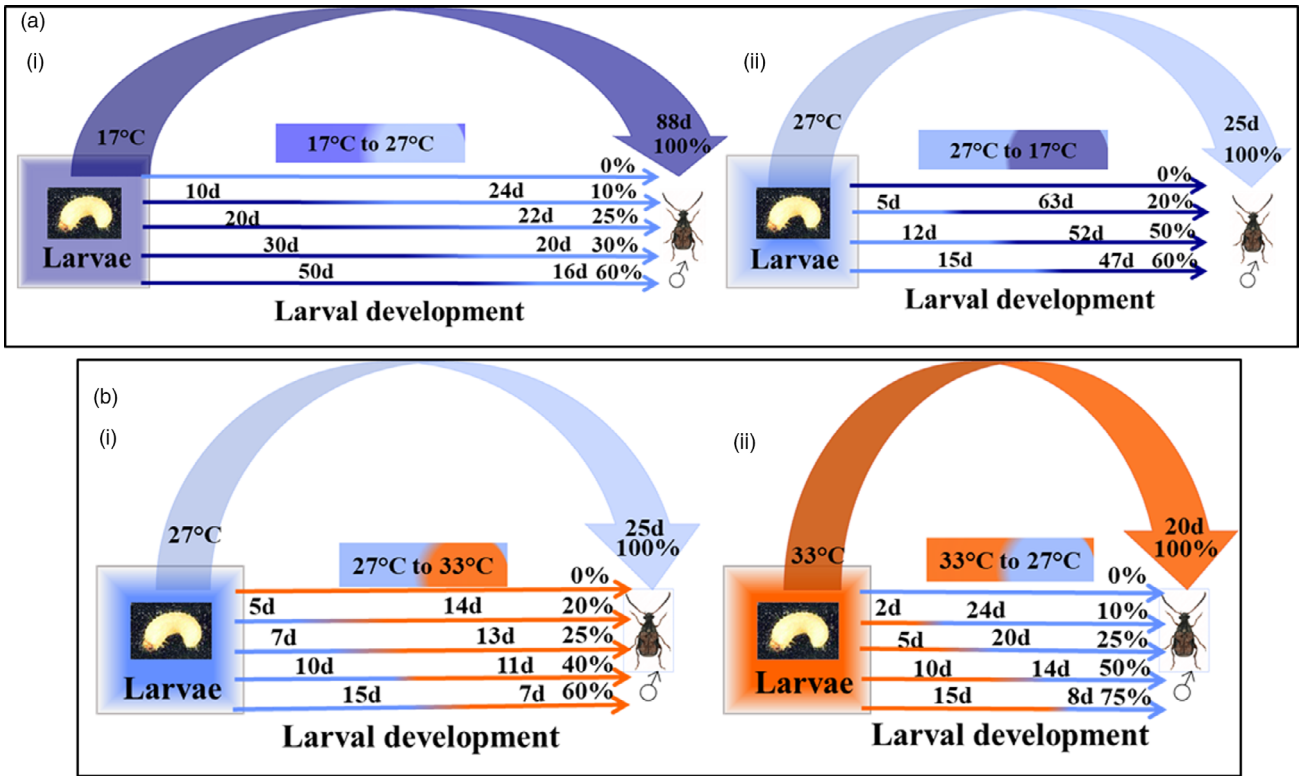

Figure 1 Protocol describing the two temperature switches relative to 100% larval development. Panel (a) (i and ii) indicate temperature switches (in days) during larval development between 17°C to 27°C and 27°C to 17°C, indicated by dark blue (17°C) and light blue (27°C) gradient lines and the direction of the temperature switch. Panel (b) (i and ii) represents switch protocol between 27°C to 33°C and the reciprocal 33°C to 27°C (light blue; 27°C and orange; 33°C). The percentages indicate the switch from the focal temperature to the novel temperature alongside a subset of individuals that remained un-switched (relative to 100% larval development in days).

were dissected and ten individual sperm per male were measured per temperature switch. Normal mature sperm of *C. maculatus* consists of an elongated tail attached to a hooked head. A mitochondrial derivative is coiled along the entire length of the tail (Eady, 1994).

Statistical analysis

All data were analysed in R.3.6.1 (R Development Core Team, 2017) using R-Studio 1.2.5042 (RStudio Team, 2020), with '*car*' (Fox & Weisberg, 2019), '*tidyverse*' (Wickham 2017, Wickham *et al.,* 2019), '*rstatix*' (Kassambara, 2020a), '*broom*' (Robinson & Hayes, 2020), '*ggpubr*' (Kassambara, 2020b), '*emmeans*' (Lenth, 2020), '*effects*' (Fox & Weisberg, 2019) and '*Rmisc*' (Hope, 2013) for data exploration, predictor effects and treatment group and textual summaries. All the graphs were created using '*ggplot2*' package in R (Wickham, 2011). We first checked whether the relationship between the variance and the mean of the response variable along with the assumptions for the best fit of the model to the data to determine the relevant error distribution (Crawley, 2006). Data were analysed using linear mixed models (LMMs) in '*lme4*' package (Bates *et al.,* 2015) and models were fitted using Restricted Maximum Likelihood (REML) in order to allow for model refinement and validation (Thomas *et al*., 2013). Violations of the assumptions of normality and homoscedasticity were checked using the residuals from linear models (Thomas *et al*., 2013). Significance of fixed effects in LMMs was obtained using F-tests with Satterthwaite's approximation for degrees of freedom implemented in '*lmerTest*' (Kuznetsova *et al*., 2017).

Post hoc analyses were performed in '*multcomp*' and '*emmeans*' to get specific pairwise comparisons between the different developmental stages (Hothorn *et al*., 2008). While building the statistical models, developmental switch time-points ('Day') were entered as fixed factors in the form of percentage of development time at the original temperature (e.g. 0%–100%). Ten randomly chosen replicate sperm-length measurements from the same male were used. Replicate male ID (i.e. 'MaleID') was entered as a random factor within the model. Testes size area (mm^2) was quantified by following a previously published method (Gay *et al*., 2009). As the lobes were approximately spherical, their two-dimensional area is likely to reflect their three-dimensional volume (Gay *et al*., 2009). Finally, allometric effects were checked by computing relative testes size and sperm length after controlling for elytra length (mm), a proxy for body size, using analysis of covariance (ANCOVA).

Results

17°C switched to 27°C and 27°C switched to 17°C: Testes size and sperm length

The absolute testes size of imagoes that completed 100% of their larval development at 17°C or 27°C were statistically similar (Fig. 2a; $z = -0.30$, $P = 1.00$). In the 17°C to 27°C switch, elytra length (see Table S1) did not explain a significant amount of the variation in testes size ($\beta = 0.01$; 95%CI: −0.08–0.12; $F_{1,52} = 0.07$, $P = 0.78$) and was thus dropped from the model. Switching larvae from 17°C to 27°C affected overall testes size (β = 0.19; 95%CI: 0.17–0.22; $F_{5,45} = 9.1$, $P < 0.0001$, Fig. 2a), with two apparent sensitive phases; one early on in development (0–25% approximately) and one later in development (between ~40% and 60%). When switched from 27°C to 17°C, the pattern was less clear. Elytra length was a significant covariate and was thus retained in the model ($\beta = 0.09$; 95%CI: 0.0006–0.18; $F_{1,44} = 4.12$, $P = 0.048$), with switch time also affecting testes size ($F_{4,44} = 7.1$, $P < 0.0001$, Fig. 3a). From these results, it is suggested that testes size was temperature-sensitive between ~25%–60% of larval development for the 27°C to 17°C temperature switch.

Sperm length was longest when approximately 90% of larval development took place at 27°C mean = 0.175 ± 0.001 (mm, 95%CI). By contrast, sperm length was smallest when ~80% of larval development took place at 17°C mean=0.157 ± 0.001 (mm, 95%CI). In the 17°C to 27°C temperature switch (Fig. 2b, blue line, right panel), sperm length was not related to elytra length (β = 0.00001; 95%CI: −0.01–0.01; $F_{1,53} = 0.0001$, $P = 0.9$, Table S1) but did vary according to when larvae were switched from 17°C to 27°C ($\beta = 0.17$; 95%CI: 0.15–0.19; $F_{5,45} = 17.8$, $P < 0.0001$, Fig. 2b). *Post hoc* testing revealed that spending the first 10% to 50% of development at 17°C before being switched to 27°C had little impact on sperm length, with sperm length being more reflective of development at 27°C. However, when larvae were switched from 17°C to 27°C after ~50% of development at 17°C, sperm length declined (Fig. 2b). Where development started at 27°C and was switched to 17°C, elytra length had no effect on sperm length ($\beta = -0.0001$; 95%CI: −0.01–0.01; $F_{1,44=}0.0006$, $P = 0.9$). The timing of the switch again significantly affected sperm length ($\beta = 0.16$; 95%CI: 0.13–0.18; $F_{4,36=}16.48$, $P < 0.0001$, Fig. 2b). *Post hoc* analyses revealed switching from 27°C to 17°C after ~40% of development resulted in a sperm length more typical of a beetle completing 100% of its development at 27°C. If the switch occurred earlier in development (i.e. the majority of larval development was at 17°C) then sperm length was more typical of those larvae completing 100% of development at 17°C. This suggested a sensitive phase in sperm length expression between 40–60% of larval development.

27°C switched to 33°C and 33°C switched to 27°C: Testes size and sperm length

In the 27°C to 33°C switch, testes size was not related to elytra length ($\beta = 0.03$; 95%CI: −0.07–0.14; $F_{1,53} = 0.48$, $P = 0.48$) whilst switching temperature affected testes size ($\beta = 0.19$; 95%CI: 0.16–0.21; $F_{5,45} = 3.5$, $P < 0.01$, Fig. 3a). *Post hoc* tests revealed that testes size was sensitive to elevated temperature during the early stages of development, approximately 20% of development. After that, testes size was relatively fixed in the face of a temperature increase from 27°C to 33°C. A similar pattern is revealed in the reciprocal switch, 33°C to 27°C: Testes size was unaffected by elytra length ($\beta = 0.01$; 95%CI: −0.08–0.10; $F_{1,53} = 0.04$, $P = 0.83$) but was affected by switching temperature ($\beta = 0.24$; 95%CI: 0.22–0.27; $F_{5,45} = 7.0$, $P < 0.0001$, Fig. 3a), with the greatest change, is testes size being apparent when the switch took place early on during development, around 10–25% of development time. Switching from 33 to 27°C after 25% of development time had little effect on testes size.

Sperm length was greatest when larvae completed 100% of their development at 27°C (mean = 0.172 mm ± 0.002 95% CI) and was shortest when larvae spent ~80% of their development at 33°C (mean=0.151 mm ± 0.001 95%CI). In both the 27 to 33°C and the 33 to 27°C switch, sperm length was unrelated to elytra length ($\beta = 0.004$; 95%CI: −0.02–0.01; $F_{1,53} = 0.22$, $P = 0.64$ and $\beta = -0.005$; 95%CI: −0.02–0.01; $F_{1,53} = 0.49$, $P = 0.48$). When switching from 27°C to 33°C sperm length was significantly affected by switching temperature ($\beta = 0.16$; 95%CI: 0.15–0.16; $F_{5,54} = 12.1$, $P < 0.0001$, Fig. 3b). Larvae that experienced early stages of development at 27°C (the first 5 or 7 days or ~20%–25% into development) had sperm lengths that resembled males that spent 100% larval development at 33°C. In the reciprocal switch (a starting temperature of 33°C and dropped to 27°C), switching the temperature significantly affected sperm length ($\beta = 0.17$; 95%CI: 0.17–0.17; $F_{5,45} = 7.7$, $P < 0.0001$, Fig. 3b). When larvae spent the first 10% of their development at 33°C (with the remaining time spent at 27°C), sperm length was reminiscent of those males reared for their entirety at 27°C. Switching temperature after ~25% of development at 33°C resulted in sperm length more closely resembling that of males that experienced 100% of their development at 33°C. This indicated that sperm length is most sensitive to temperature early on during larval development.

Discussion

Testes size and sperm length (primary sexual traits) in imagoes were influenced by the temperature experienced at earlier developmental stages (also see, Blanckenhorn & Hellriegel, 2002; Minoretti *et al*., 2013; Breckels & Neff, 2014; Vasudeva *et al*., 2014; Reinhardt *et al*., 2015; Iossa *et al*., 2019; Vasudeva *et al*., 2019). Using a temperature-switch protocol, we show plasticity in sperm length and testes size to be age-specific and that periods of temperature sensitivity depended on the direction of the temperature switch, up or down, from normal (27°C). In the 17°C–27°C switch, sperm length sensitivity was observed in the second half of larval development, whilst in the 27°C–33°C switch, sensitivity was observed in the first half of larval development. Testes size plasticity was also sensitive to temperature switching during early developmental stages in the 27°C–33°C switch, although patterns of testes size plasticity were much less clear during the 17°C–27°C

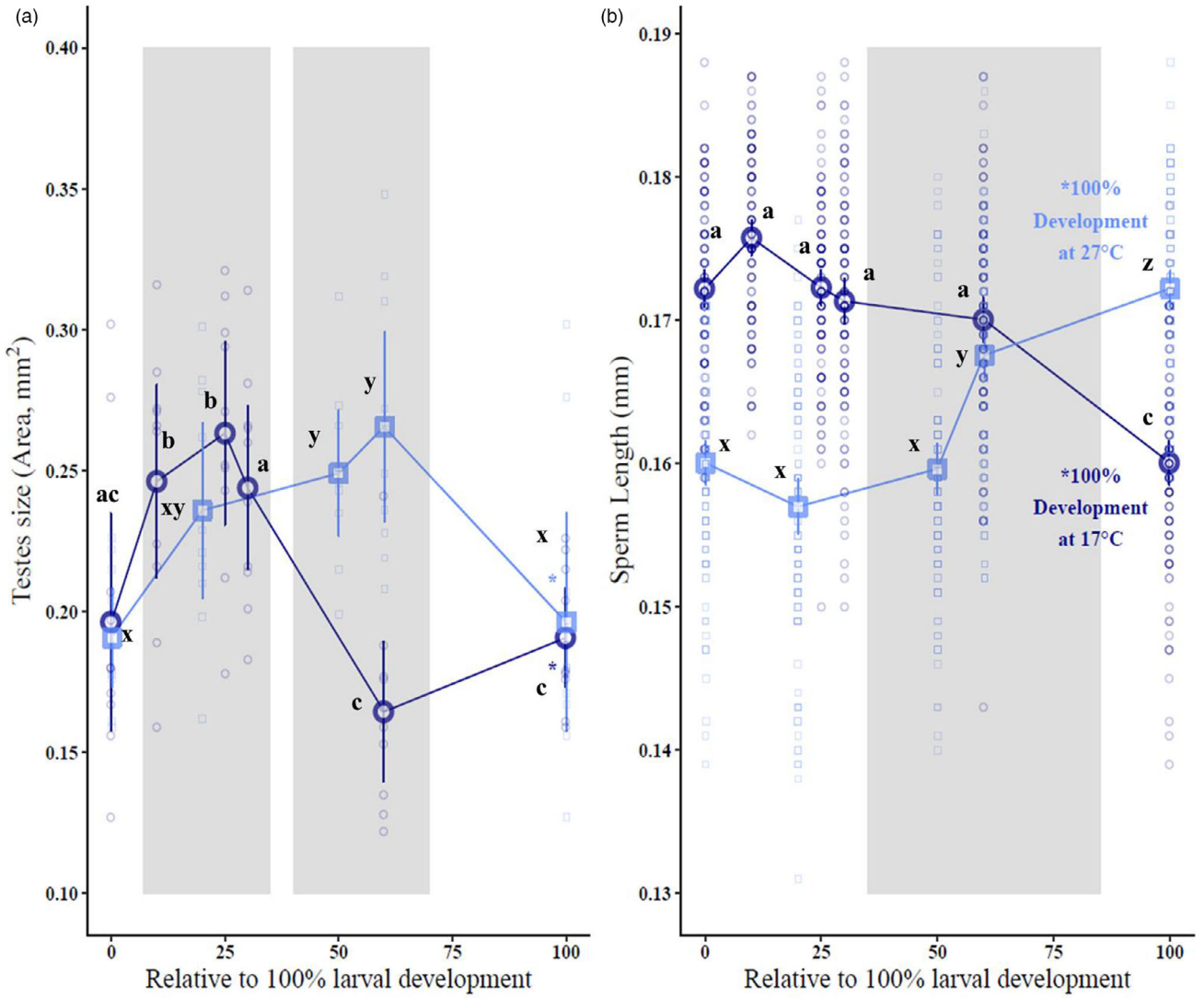

Figure 2 Line plots of testes size (mm^2, ± 95%CI) and sperm length (mm, ± 95%CI) following temperature switch from 17°C to 27°C (dark blue line; circle points) and 27°C to 17°C (light blue line; square points). Shaded area represents larval stages experiencing temperature sensitivity in the expression of testes size (a) and sperm length (b). Points denoted by different alphabets are trait mean values that are statistically significantly different for larval developmental stages that experienced temperature switch. Asterisks indicate trait means through 100% larval development. Body size had a marginally significant effect on testes size when switched from 27°C to 17°C (see Results).

switch and appeared to depend on whether the switch was from 27°C to 17°C or 17°C to 27°C.

Previous work using *C. maculatus* has shown males experiencing relatively high (33°C) or low (17°C) developmental temperatures produce smaller sperm than males grown at intermediate temperatures (Vasudeva *et al.*, 2014). Males producing small sperm were less successful in sperm competition assays than their longer-sperm rivals, although other confounding factors (e.g. sperm number) could account for the observed differences in sperm competition outcome. Indeed, it is likely that both the quantity and quality of sperm and seminal fluid were altered by the temperature regime experienced. A further variable that is very likely to impact sperm function, is the female reproductive environment (Eberhard, 1996; Wilson *et al.*, 1997) and in the sperm competition assays of Vasudeva *et al.*, (2014) sperm competition was played out in females reared at a standard 27°C environment.

Sperm length exhibits remarkable interspecific variability (e.g. a 100-fold variation within the Drosophila genus; Pitnick *et al.*, 1995) and it is considerable within species variation (reviewed in Snook, 2005). In *C. maculatus*, mean sperm length has been reported as small as 0.157 mm in Gay *et al.*, (2009) and up to 0.177 mm in Rugman-Jones and Eady (2007), with consistent between male variability reported by Dowling *et al.*

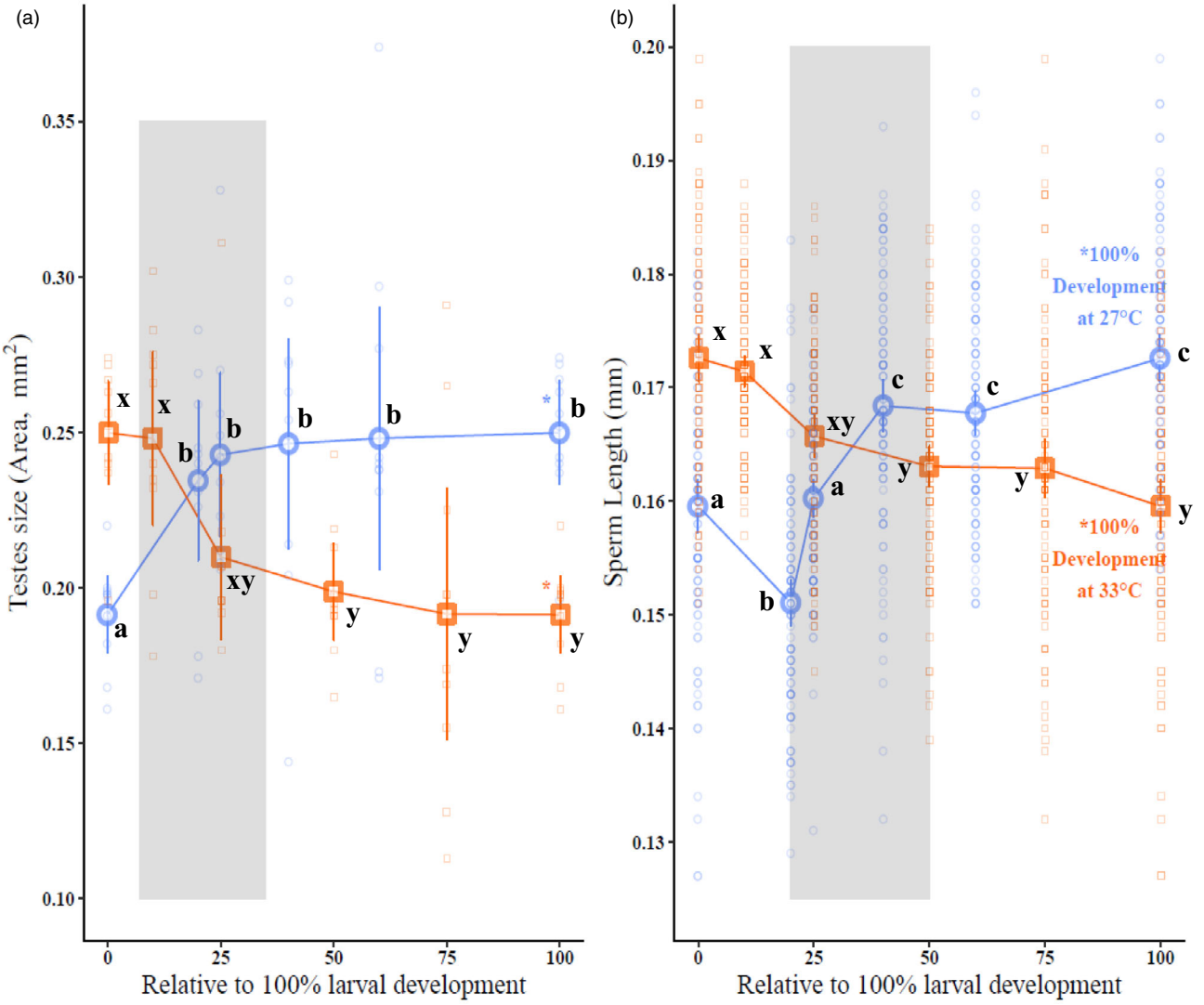

Figure 3 Line plots of (a) testes size (mm^2, ± 95%CI) and (b) sperm length (mm, ± 95%CI) following temperature switch from 27°C to 33°C (light blue line; circle points) and 33°C to 27°C (orange line; square points). Shaded area within the two boxes represents larval stages experiencing temperature sensitivity in the phenotypic expression of reproductive traits. Larval stages denoted by different alphabets are statistically significant. Asterisks indicate trait mean values for 100% larval development. Body size did not influence the expression of both testes size and sperm length across both temperature switches.

(2006). The functional significance of sperm length has been explored in relation to the evolution of sperm flagellum and mid-piece length in vertebrates, linking it to faster swimming abilities in response to sperm competition (Fitzpatrick *et al.*, 2009; Lüpold *et al.*, 2009; Gómez Montoto *et al.*, 2011; also see Lüpold *et al.*, 2020a). Furthermore, some comparative studies reporting an association between sperm competition risk and sperm length (LaMunyon & Ward, 1999; Morrow & Gage, 2000; Byrne *et al.*, 2003), although other studies found no association (Hosken, 1997; Rugman-Jones & Eady, 2007). Within species, Gage & Morrow (2003) showed that in *Gryllus bimaculatus* smaller sperm achieved greater fertilization success under sperm competition. By contrast, in *T. castaneum,* males evolved longer and more competitive sperm after 77 generations of selection when evolving under elevated levels of sperm competition compared to males evolving under low levels of sperm competition (Godwin *et al.*, 2017). In *D. melanogaster*, the relationship between sperm length and function is further complicated when the reproductive architecture of the female is considered. Following artificial selection for long and short sperm in males and long and short seminal receptacle (SR) length in females, Miller & Pitnick (2002) showed that long-sperm males outcompeted short-sperm males when the competition took place in females with long SRs. There was no difference in sperm competitive advantage (P_2) between the long and short sperm when sperm competition took place within short

SRs (also see Lüpold *et al.,* 2016, Lüpold, 2020b for confirmation). Furthermore, in the zebra finch (*Taeniopygia guttata*), Bennison *et al.,* (2015) found males with longer sperm to outcompete males with shorter sperm, whilst in the dung beetle (*Onthophagus taurus*), García-González & Simmons (2007) found fertilization success was biased towards males with shorter sperm. This suggests that under conditions of sperm competition, females exert considerable selection on sperm length. Taken together, these studies indicate that we are still some way from a universal understanding of sperm length variation.

In ectotherms, temperature affects the rate of biochemical reactions and the fluidity of cellular and organelle membranes, altering the transport of molecules in to and out of cells (Neven, 2000; Angilletta Jr., 2009) affecting higher levels of biological organization. These effects can manifest themselves as changes to organismal function in the short-term (seconds/minutes), medium-term (hours/days), or the long-term across ontogenetic stages (Colinet & Hoffmann, 2012; Abram *et al*., 2017). In terms of ontogeny, there is substantial plasticity in gene expression in response to temperature (Galarza *et al*., 2019), which can result in altered patterns of resource allocation that affect subsequent stages. These effects can be beneficial, through the production of alternative morphs (polyphenisms) and/or through hardening (developmental acclimation: Colinet & Hoffmann, 2012; Schiffer *et al*., 2013). Alternatively, the effects can result in carry-over effects that negatively impact the adult phenotype, which in turn, can have knock-on effects at higher levels of biological organization (Harrison *et al*., 2011). The general decline in male fertility in response to thermal stress during development suggests that some of these carry-over effects are negative (O'Connor *et al*., 2014; Porcelli *et al*., 2017; Walsh *et al*., 2019; Parratt *et al*., 2020). However, in *T. castaneum*, within generation thermal plasticity in sperm size has been shown to be adaptive: Females mated to experimental males derived from pupae held at either 30 or 38°C produced most offspring, when oviposition took place at the same temperature as that experienced by the male pupae (Vasudeva *et al*., 2019). Such positive carry-over effects might play a crucial role in fertilization success under global climate change.

Our results suggest plasticity in sperm length is associated with two phases of development depending on whether the temperature was switched up or down. Bowler and Terblanche (2008) suggest different mechanisms may operate depending on whether the organism faces heat stress or cold stress. Low-temperature tolerance appears to be influenced by a host of hierarchical events, including reduced functioning of Na^+/K^+ pumps in critical tissues that affects the ability to regulate ion balance. By contrast, it has been argued that heat tolerance is controlled by fewer hierarchical events, being mainly a product of cellular-level effects (Bowler & Terblanche, 2008). Thus, different processes operating at different times could potentially result in two sensitive phases.

These different thermal response pathways could act on different developmental stages. The early stages of larval development tend to be associated with gonadal differentiation whilst the latter stages of larval development are associated with testicular differentiation and spermatogenesis (Jacob, 1989; Friedländer *et al*., 2005). In *Drosophila,* primordial germ cells and somatic precursor cells coalesce to form an early gonad around stage 12–14 (~12 h following fertilization), with testes development largely complete by the end of embryogenesis (Clark *et al*., 2007; Whitworth *et al*., 2012). This process requires the activation of a series of complex and sequential control mechanisms that are likely to be highly susceptible to environmental perturbation. Thus, thermal stress applied at this time could disturb normal testicular development, ultimately disrupting spermatogenesis later in ontogeny. For example, in the stick insect, *Carausius morosus*, heat stress applied during the early stages of embryogenesis can disrupt sexual differentiation, increasing the incidence of masculinized females (Berg-erard, 1972). Similar temperature-dependent sexual differentiation is apparent in some high-latitude mosquito larvae: Male larvae reared at higher than normal temperatures can develop ovaries and other female characteristics (Horsfall & Anderson, 1964). These examples illustrate that temperature can impact gonadal morphogenesis and it is likely that these environmental stressors will have lasting impacts on spermatogenesis throughout later instars.

Heat stress applied at later developmental stages is likely to disrupt the actual process of spermatogenesis. In the locust, *Locusta migratoria,* sub-optimal temperatures applied to the imago rendered males infertile, primarily brought about by the asynchronous development of germ cells within cysts, abnormal development of sperm cell organelles, reduced spermatid elongation and an inability to form sperm bundles (Szöllösi, 1976). Similar effects of developmental temperature on cyst elongation have been reported in *Drosophila* (David, 1971; Cohet, 1973). High temperatures during development resulted in an increase in spermatid death in the cysts, abnormalities in chromatin condensation, the abnormal positioning of sperm heads within the cyst and impairment of cyst elongation and by extension spermatid elongation (Joly *et al*., 1989; Rohmer *et al*., 2004). These cyst-related aberrations most likely prevent the complete maturation of sperm which subsequently prevents the migration of sperm from the testes to the seminal vesicle (David *et al*., 2005). Presence of motile sperm within the seminal vesicle is a key indicator of male fertility (Chakir *et al*., 2002; Porcelli *et al*., 2017).

Relatively few studies have examined the effect of heat stress applied to multiple stages of larval development and fewer still the effects of stage and temperature on subsequent reproduction. Those that have, tend to focus on female fecundity. For example, Zhang *et al*., (2015) reported the effects of heat shock applied at various developmental stages on female fecundity in the moth, *Plutella xylostella*. They found that thermal stress applied to stages from the egg to the adult resulted in decreased fecundity and that heat stress applied closer to the imago resulted in the greatest effect. (Ma *et al*., 2004) reported similar findings in the rose grain aphid, *Metopolophium dirhodum*. To our knowledge, similar studies on male reproductive parameters have yet to be published, although it is well established that heat stress applied prior to the imago stage negatively impacts sperm form and function (Sales *et al*., 2018; Walsh *et al*., 2019).

It is becoming increasingly clear that thermal stress impacts fertility (especially of males) before critical lethal temperatures are reached (Walsh *et al*., 2019; Parratt *et al*., 2020). Recent models indicate that patterns of species distribution are better predicted by thermal fertility limits than lethal temperature limits and that thermal-fertility limits have important consequences for models of population persistence in the face of global warming (Parratt *et al*., 2020). That we show temperature and developmental stage to interact in their effects on the form and function of 'primary' reproductive traits. Most populations comprise individuals at different developmental stages and thus a transient, hot event could have a disproportionately large (or small) effect on the fertility of the different developmental stages. Thus, those individuals that find themselves serendipitously at less thermally sensitive stages of development are likely to be able to compensate for the loss of fertility experienced by those encountering thermal stress at more developmentally sensitive stages.

Variation in the ability of individuals to buffer against thermal stress during development opens the door for condition-dependent sexual selection to increase the rate of adaptation in response to climate change (Cally *et al*., 2019). Females that mate with males better able to buffer their development against thermal stress will have enhanced fertility themselves. This process could be further augmented by the fact that males experiencing thermal stress during development are less likely to mate with females (Vasudeva *et al*., 2018; Iossa *et al*., 2019; Sutter *et al*., 2019) and, if they do, are more likely to lose out in terms of sperm competition (Vasudeva *et al*., 2014). However, this scenario ignores the impact of thermal stress on female reproductive form and function (Iossa, 2019). There is growing evidence that temperature stress during development affects the female reproductive phenotype (Fischer *et al*., 2003; Stillwell & Fox, 2005; Liefting *et al*., 2010; Guillaume *et al*., 2016; Sgrò *et al*., 2016), altering the 'rules of the game' by which post-copulatory sexual selection is played out (Eberhard, 1996; Farrow *et al.,* 2020, *submitted*). Thus, exactly how post-copulatory sexual selection will play out in the face of environmental change remains to be fully explored.

In conclusion, we show that the thermal tolerance of spermatogenesis is not static over the physiological age of the insect, but rather changes in an age-dependent and stress-dependent manner. We believe this approach will help identify those ontogenetic stages most at risk in the face of global warming and that through studying stage-specific sensitivities, we will have a better understanding of the mechanisms underlying the loss of male fertility in response to thermal stress.

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Data Availability Statement

All data generated, analysed in this study along with the annotated R codes, are made openly available as an associated source file in our Mendeley data repository with the https://doi.org/10.17632/rsjcmdwby7.3 (under CC BY 4.0 licence) (Vasudeva *et al*., 2021). The complete data set will be publicly accessible after the embargo period, from 3rd March 2021 using the DOI mentioned above.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Body size (elytra length, mm), testes size (mm2) and sperm length (mm) variation in response to temperature switch experiments.